

Horizontal Transmission of *Amblyospora albifasciati* García and Becnel, 1994 (Microsporidia: Amblyosporidae), to a Copepod Intermediate Host and the Neotropical Mosquito, *Aedes albifasciatus* (Macquart, 1837)

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The life cycle of *Amblyospora albifasciati* is characterized by three sporulation sequences involving the definitive mosquito host and a copepod intermediate host. Meiospores of *A. albifasciati* were infectious *per os* to adult females of the copepod *Mesocyclops annulatus*. All developmental stages in the copepod had unpaired nuclei, with sporulation involving the formation of a sporontogenic interfacial envelope and the production of a second type of uninucleate spore. These spores, formed in the ovaries of *M. annulatus*, were large, pyriform, and measured $10.4 \times 4.8 \mu\text{m}$. They infected *Aedes albifasciatus* larvae when ingested to initiate a sequence that involves schizogony and gametogony and ends with plasmogamy and nuclear association to form diplokaryotic meronts. Oval binucleate spores ($9.3 \times 3.1 \mu\text{m}$) are formed in the adult mosquito and are responsible for vertical transmission to the filial generation. © 2000 Academic Press

Key Words: *Amblyospora albifasciati*; Microsporidia; *Aedes albifasciatus*; mosquito; *Mesocyclops annulatus*; copepoda; taxonomy; ultrastructure; host specificity.

INTRODUCTION

Aedes albifasciatus (Macquart, 1837) is a common flood-water mosquito that is widely distributed in freshwater habitats throughout Argentina (Darsie and Mitchell, 1985). At high population densities it is an important pest in urban areas and contributes to economic losses in the dairy and beef industry (Ludueña Almeida, 1994). In addition, Avilés *et al.* (1992) demonstrated that *A. albifasciatus* is a potential vector of western equine encephalitis virus.

Several parasites and pathogens were reported as potential regulators of *A. albifasciatus* by Maciá *et al.* (1995). One of these natural enemies, *Amblyospora albifasciati* García and Becnel, 1994 was described based on the morphology and ultrastructure of the

meiospores (García and Becnel, 1994). These authors speculated that *A. albifasciati* is vertically transmitted by binucleate spores in adult female mosquitoes and that a spore formed in an intermediate host is responsible for horizontal transmission to the mosquito host (Sweeney *et al.*, 1985, 1988, 1990; Andreadis, 1985a,b, 1988; Becnel, 1992; White *et al.*, 1994). This investigation identifies the intermediate host of *A. albifasciati* as *Mesocyclops annulatus* (Wierzejski, 1892), and demonstrates horizontal transmission with spores formed in the copepod host to larvae of *A. albifasciatus*. These two new developmental sequences are described with evidence presented to document vertical transmission.

MATERIALS AND METHODS

Collection Site

Aedes albifasciatus larvae were collected from an open pool ($30 \times 15 \times 0.30 \text{ m}$) near La Plata, Buenos Aires province, Argentina. The breeding site was not connected with any permanent bodies of water and was intermittently flooded by rain. Adults were collected near the larval breeding site. The study area was subjected to drying during the months of May–August and November–February.

Collection and Processing of Mosquito Larvae

Mosquito larvae infected with *A. albifasciati* were collected in September and October 1995 and again in March and April 1996. Infected larvae were identified according to keys of Lane (1953) and Darsie and Mitchell (1985). Pieces of live infected larvae were smeared on microscope slides, air dried, fixed in methanol for 3 min and stained for 10 min with 10% Giemsa-stain solution buffered at pH 7.41.

Collection and Processing of Mosquito Adults

Mosquito adults were collected in March and April 1996 with a battery powered aspirator. Females were fed on a restrained chicken placed directly into the cage. One hundred engorged females were held individually in vials and allowed to oviposit on filter paper. After oviposition, the females were checked for infection by examination of fresh squashes for the presence of spores. Eggs from individual females were hatched and placed into containers with 500 ml of water and held at 26°C. Larvae were fed ground fish food and examined for patent fat body infections as fourth instars.

Collection and Processing of Copepods

Copepods were collected from the same site and identified to species according to Ringuelet (1958), Dussart (1969), and Reid (1985). Gravid females from each species found at the site were isolated and placed in 100 ml of water. Fish food was added for nutrition. The progeny of these females were maintained in containers with 700 ml of water for transmission experiments with meiospores of *A. albifasciati*.

Copepods collected during the study were examined microscopically to detect patent infections. Infected copepods were fixed in 2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate containing CaCl_2 (1 mg/ml) for 2 h. The copepods were postfixed in 1% OsO_4 (w/v) for 2 h, dehydrated through a graded ethanol series into acetone, and embedded in Epon-Araldite. Sections were poststained in methanolic uranyl acetate followed by Reynolds lead citrate and examined and photographed with a Hitachi H-600 electron microscope at 75 kV.

Exposure of Copepods and Mosquitoes

Mosquito to copepod transmission. Mosquito larvae infected with meiospores of *A. albifasciati* (field-collected) were triturated in water with a glass tissue grinder and then filtered through cotton. The suspension was centrifuged for 10 to 30 min at 5000 rpm. The supernatant was discarded and the meiospores were resuspended in distilled water. Spore concentrations were determined with a hemocytometer.

Copepods were divided by age (copepodid and adults) and sex and then separated into groups of 20 to 25 individuals in 10 ml of water. Each group was inoculated with meiospores of *A. albifasciati* at a final concentration of 1×10^3 spores per ml for 24 h. They were then transferred to 100 ml water and an appropriate amount of fish food. Control groups were prepared in a similar manner but without the addition of meiospores.

Copepod to mosquito transmission. Spores experimentally produced in *M. annulatus* were used as inoculum. Fifty first-instar larval *A. albifasciatus* (field collected) were placed into petri dishes containing 10

ml of water with one or two dead infected copepods. After 24 h of exposure, the larvae were transferred to containers with 750 ml of water plus fish food. Control groups were handled in a similar manner but without the addition of spores.

RESULTS

Copepod Field Studies

The three major copepod species collected from the breeding site were *Microcyclops* sp., *Mesocyclops annulatus*, and *Acanthocyclops robustus* (Sars, 1863). Microsporidia were present in all three species during the sampling period. The most commonly found microsporidium was an undescribed *Tuzetia*-like species. *Mesocyclops annulatus* was the only copepod with spores resembling those commonly associated with *Amblyospora* spp. The natural prevalence of patent infections of *M. annulatus* with *A. albifasciati* was 0.75% (16/2120) and these were found during the spring (September and October). Males were not infected and infected females were never found to be gravid (egg bearing).

Infections in Field Collected *A. albifasciatus* Adults

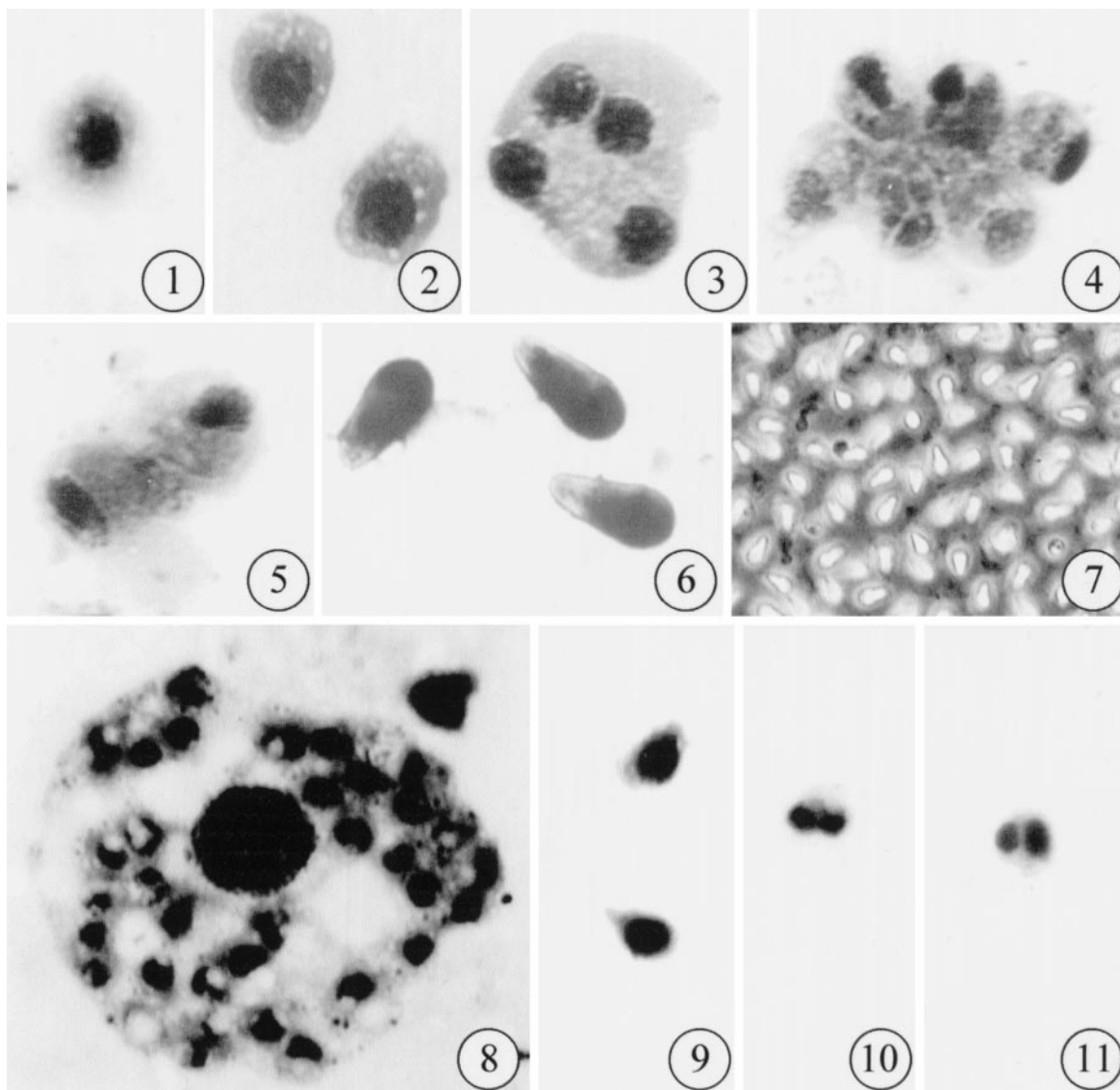
The prevalence of infection in adult females collected in March and April was 1% (1/100). Spores were formed after a blood meal and were localized in the ovaries of the infected female as determined by dissection. The binucleate spores were oval in shape and measured $9.27 \pm 0.87 \times 3.10 \pm 0.3 \mu\text{m}$ (fresh, $n = 15$). This infected female laid 181 eggs of which 120 hatched. Percentage patent infection (fat body infections with meiospores) was 11.6% (14/120). The sex of the infected progeny was not determined.

Laboratory Studies

Amblyospora albifasciati was successfully transmitted to adult female *M. annulatus* with infection rates between 38.9% (7/18) and 56% (14/25). Male and copepodid stages were not infected. Uninucleate spores were formed between 11 and 14 days postexposure. *Amblyospora albifasciati* was not transmitted to *A. robustus*. A culture of the *Microcyclops* sp. could not be established and was therefore not tested.

Development in the Intermediate Host *Mesocyclops annulatus*

Schizogony. Uninucleate developmental stages of *A. albifasciati* were first observed in ovaries 8–9 days postexposure. These initial stages (schizonts) were round to oval (Fig. 1) with multiplication primarily by binary fission. Schizonts were bound by a simple plasmalemma in direct contact with the host cell cytoplasm (Fig. 12). Numerous ribosomes and arrays of endoplasmic reticulum were present in the cytoplasm of the



FIGS. 1-7. Developmental stages of *Amblyospora albifasciati* from the ovarian tissue of the copepod *Mesocyclops annulatus*. **FIGS. 1-6**, Giemsa-stained, $\times 2000$; **FIG. 7**, fresh, $\times 800$. (1) Uninucleate schizonts. (2) Transitional forms. (3) Quadrinucleate plasmodium. (4) Multiple fission of sporogonial plasmodium with 6 nuclei. (5) Sporont dividing to form two sporoblasts. (6-7) Uninucleate spores. **FIGS. 8-11.** Developmental stages of *Amblyospora albifasciati* in the pupae of *Aedes albifasciatus* initiated by uninucleate spores formed in *Mesocyclops annulatus*. Giemsa-stained; $\times 2000$. (8) Host cell infected with uninucleate stages of *Amblyospora albifasciati*. (9) Gametes. (10) Fusion of gametes. (11) Diplokaryotic meront.

schizont. The nucleus occupied a large portion of the cell and was commonly observed with spindle plaques apparently in preparation for division (Fig. 12).

Transition to sporogony. Stages believed to be transitional were larger than the schizonts with a slightly vacuolated cytoplasm (Fig. 2). They were first distinguished ultrastructurally by thickened regions of the plasmalemma (Fig. 13).

Sporogony. Sporonts multiplied by repeated karyokinesis without cytokinesis to form sporogonial plasmodia with four to eight nuclei (Figs. 3-4). These plasmo-

dia divided by multiple fission via rosette formation to produce stages that transformed directly into sporoblasts (Figs. 4, 14). Occasionally, some of these products of multiple fission appeared to undergo an additional binary fission to produce two sporoblasts (Fig. 5). A delicate interfacial envelope developed as a separate unit membrane exterior to the plasmalemma within which an amorphous secretory material accumulated (Figs. 14, 15). This sporontogenic interfacial envelope (of parasite origin) appeared to divide with the sporogonial plasmodium to individually enclose each sporoblast (Figs. 14-16).

Spore. Early uninucleate sporoblasts were identifiable by the primordium of the polar filament and a space between the thickened plasmalemma and the interfacial envelope (Figs. 16, 17). This space increased with development and apparently represented shrinkage of the sporoblast with maturation (Fig. 18). As sporogenesis progressed, the sporoblast became elongate and large vacuoles accumulated in the anterior part of the developing spore, representing the initial stages in the development of the polaroplast (Figs. 18, 19).

The mature spore was pyriform in shape and measured $9.28 \pm 0.66 \times 4.17 \pm 0.44 \mu\text{m}$ (fixed, $n = 15$; Fig. 6) and $10.4 \pm 0.63 \times 4.8 \pm 0.12$ (fresh, $n = 15$, Fig. 7). The most notable morphological aspect of the spore was the large and compartmentalized polaroplast (Fig. 20). The spore wall was composed of an endospore that was approximately three times thicker than the thin exospore (Fig. 21). The polar filament was isofilar and made 9–11 turns in the posterior region of the spore.

Horizontal Transmission to Mosquito

Experimental transmission with spores from *M. annulatus* to larval *A. albifasciatus* revealed stages of *A. albifasciati* in Giemsa-stained smears of pupae and adults. The infection levels were low with only 3 of 31 survivors positive for developmental stages of *A. albifasciati*. Stages were commonly observed in the cytoplasm of host cells of unknown origin (Fig. 8). The earliest stages were small, uninucleate, and round in shape with relatively large nuclei (Fig. 8). Later stages (gametes) were pyriform in shape with the single nucleus located at the broad end of the cell (Fig. 9). Gametes came together in pairs (Fig. 10) and underwent cytoplasmic fusion (plasmogamy) to form cells with 2 nuclei in a common cytoplasm. These nuclei did not fuse but became associated to form a diplokaryon (Fig. 11). The resulting diplokaryotic cells are the first meronts in the cycle. Because *A. albifasciatus* has not been colonized, it was not possible to obtain progeny from exposed female adults to verify binucleate spores or vertical transmission in the lab.

DISCUSSION

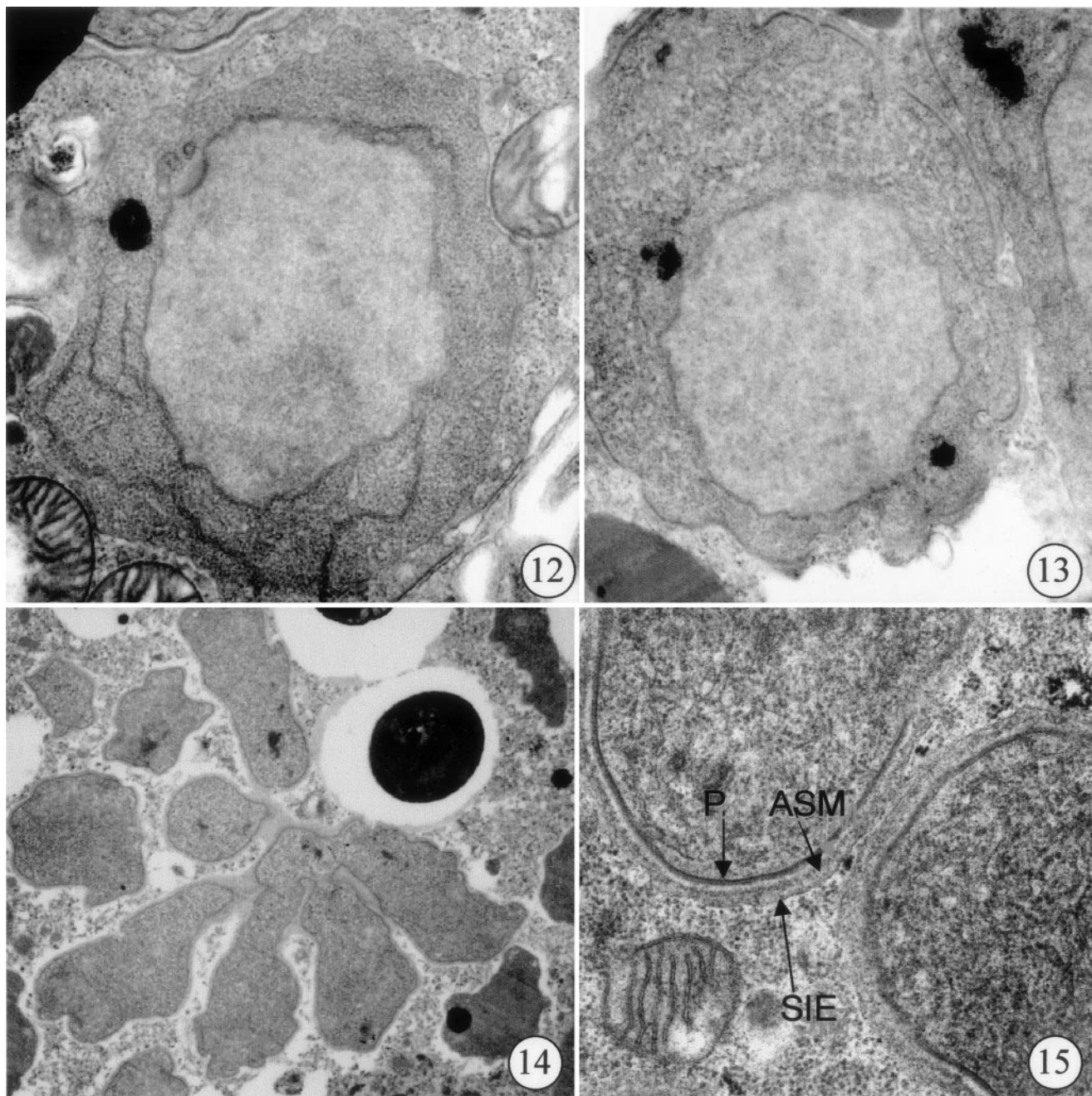
This field and laboratory study has confirmed the role of a copepod intermediate host in the life cycle of *A. albifasciati*. Meiospores of *A. albifasciati* infected the copepod *M. annulatus* and resulted in the production of uninucleate spores that are directly infectious to larvae of *A. albifasciatus*. Because this mosquito has not been colonized, the part of the life cycle of *A. albifasciati* that involves vertical transmission could not be determined in the laboratory. However, vertical transmission of *A. albifasciati* was verified with field collected adults of *A. albifasciatus*. Therefore, the life cycle of *A. albifasciati* is essentially identical to that described for other

species of *Amblyospora* (Andreadis, 1988; Sweeney *et al.*, 1988, 1990; Becnel, 1992; White *et al.*, 1994) and is characterized by three sporulation sequences involving two host species. *A. albifasciati* represents only the second species of *Amblyospora* from *Aedes* mosquitoes for which the complete life cycle has been documented (Andreadis, 1988).

The sporulation sequence of *A. albifasciati* in the copepod *M. annulatus* is similar to that reported for the type host *A. californica* (Kellen and Lipa, 1960) as described by Becnel (1992). All developmental stages in the copepod are uninucleate with sporulation involving the production of multinucleate plasmodia to produce uninucleate spores. Sporulation stages in the copepod host are contained within a nonpersistent sporontogenic interfacial envelope. The spore is characteristic of those previously described for other *Amblyospora* spp. with the most notable feature being the large, vacuolated polaroplast that occupies the majority of the spore (Andreadis, 1985a; Sweeney *et al.*, 1985, 1988, 1990; Becnel, 1992; White *et al.*, 1994; Becnel and Andreadis, 1998; Micieli *et al.*, 1998). The sequence in the mosquito host initiated by the spores formed in the intermediate host follows the pattern established in previous studies (Andreadis, 1988; Sweeney *et al.*, 1988; Becnel, 1992). In this sequence, schizogony and gametogony end with plasmogamy and nuclear association to form diplokaryotic meronts. The infected cells reported in this study probably represent oenocytes that harbor the parasite for transfer to the adult where sporulation results in the production of binucleate spores.

Field and laboratory data confirms that of the three cyclopoid species commonly associated with *A. albifasciatus* only *M. annulatus* is susceptible to *A. albifasciati*. This specificity for a single copepod intermediate host has also been found by Micieli *et al.* (1998) in *A. dolosi*, by Sweeney *et al.* (1990) in *A. indicola* and *A. dyxenoides*, and by Andreadis (1988) in *A. connecticus*. *Amblyospora californica* and *A. opacita*, however, have been shown in laboratory transmissions to have two copepod species that can serve as intermediate host (Becnel, 1992; White *et al.*, 1994).

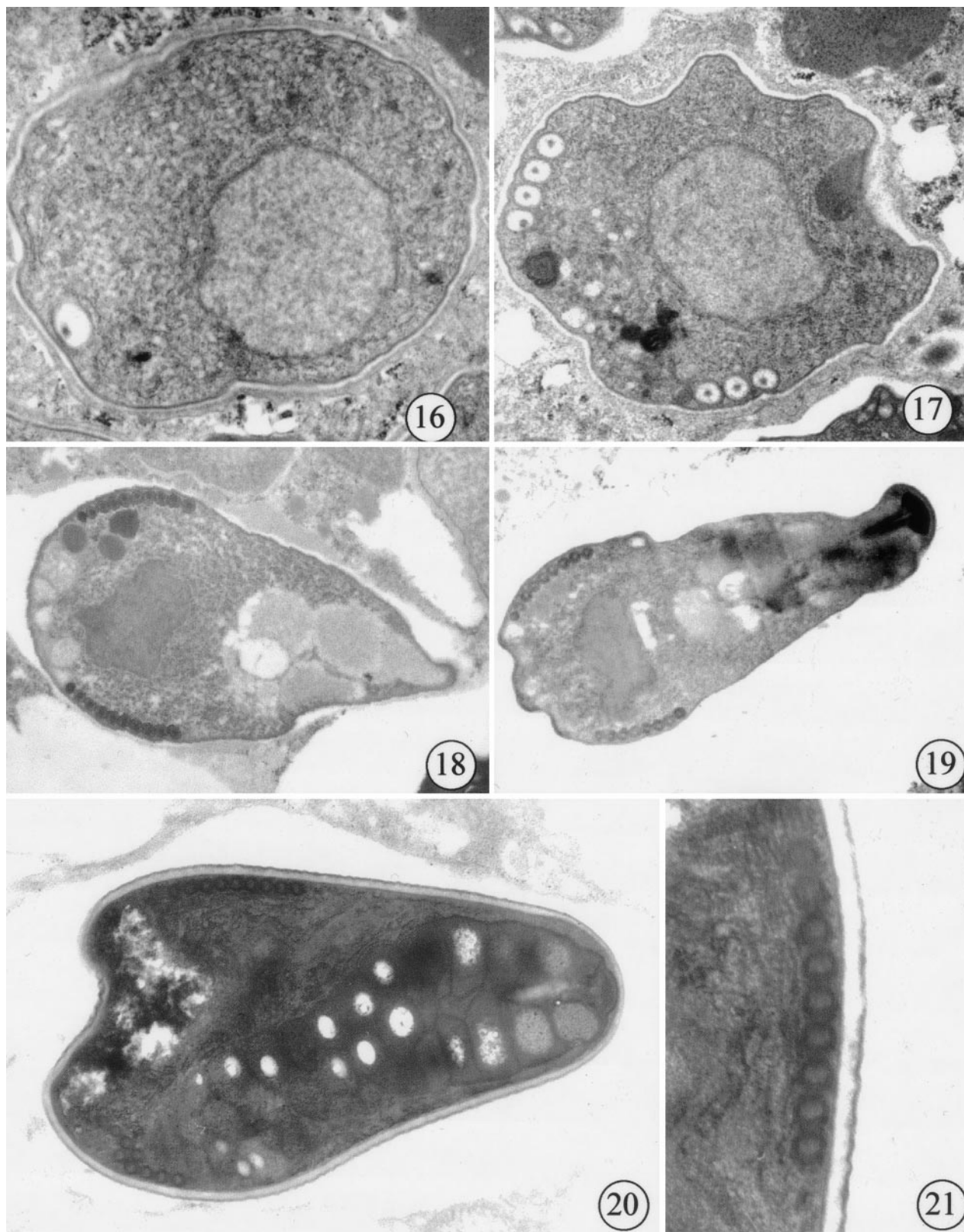
This study represents the second *Amblyospora* species from a neotropical mosquito that requires an intermediate host to complete the life cycle (Micieli *et al.*, 1998). To date, intermediate hosts have been reported for 12 species of microsporidia in the family Amblyosporidae (Table 1). This includes 10 *Amblyospora* spp. (6 from *Culex* and 4 from *Aedes*), 1 *Parathelohania* sp., and 1 *Duboscqia* sp. from *Anopheles*. While this represents but a small portion of the known species, a few general trends have emerged. Thus far, eight different genera of copepods (all members of the suborder Cyclopoida) serve as intermediate hosts and



FIGS. 12–15. Stages of *Amblyospora albifasciati* in the ovarian tissue of the copepod *Mesocyclops annulatus*. (12) Schizont $\times 31,000$. (13) Transitional form. Note the thickening of the plasmalemma which is an early indication of the transition from schizogony to sporogony; $\times 23,000$. (14) Multiple fission of a sporogonial plasmodium; $\times 7,000$. (15) Early sporoblasts enveloped by a sporontogenic interfacial envelope (SIE). The episporontal space is filled with amorphous secretory material (ASM). P, plasmalemma; $\times 40,250$.

two species, *A. vernalis* and *M. albidus*, have been shown to serve as intermediate hosts for two different *Amblyospora* spp. There does not appear, however, to be any tendency for certain genera of copepods to be associated with either a particular genus of the mosquito host or the microsporidium. For example, *Mesocyclops* spp. serve as intermediate hosts for *Amblyospora*

spp. from both *Culex* and *Aedes* mosquitoes. Likewise, *Apocyclops* spp. can serve as intermediate hosts for *Amblyospora* and *Duboscqia* spp. Information on new intermediate hosts from species of microsporidia from mosquitoes will enable further analysis on the evolutionary relationships between the parasite and its definitive and intermediate hosts.



FIGS. 16–21. Morphogenesis of the uninucleate spore of *Amblyospora albifasciati* in *Mesocyclops annulatus*. (16) Early sporoblast with remnants of the amorphous material within the episporontal space; $\times 17,700$. (17) Later sporoblast without amorphous material within the episporontal space; $\times 17,000$. (18) Immature spore; $\times 14,300$. (19) Immature spore with polar filament formation near completion and the genesis of the vacuolated polaroplast; $\times 14,000$. (20) Mature spore demonstrating the extensive polaroplast divided into numerous compartments; $\times 19,500$. (21) Details of the spore wall and cross sections of the polar filament; $\times 43,000$.

TABLE 1
Listing of Microsporidia from Mosquitoes with Copepod Intermediate Hosts

Microsporidium	Mosquito host	Copepod intermediate host	Reference
<i>Amblyospora connecticus</i>	<i>Aedes cantator</i>	<i>Acanthocyclops vernalis</i>	Andreadis, 1985a
<i>Amblyospora cinerei</i>	<i>Aedes cinereus</i>	<i>Acanthocyclops vernalis</i> ^a	Andreadis, 1994
<i>Duboscqia hilli</i>	<i>Anopheles hilli</i>	<i>Apocyclops dengizicus</i>	Sweeney <i>et al.</i> , 1993
<i>Amblyospora indicola</i>	<i>Culex sitiens</i>	<i>Apocyclops</i> sp.	Sweeney <i>et al.</i> , 1990
<i>Amblyospora stimuli</i>	<i>Aedes stimulans</i>	<i>Diacyclops bicuspidatus thomasi</i> ^a	Andreadis, 1994
<i>Amblyospora salinaria</i>	<i>Culex salinarius</i>	<i>Macrocyclus albidus</i>	Becnel and Andreadis, 1998
<i>Amblyospora dyxenoides</i>	<i>Culex annulirostris</i>	<i>Mesocyclops albicans</i>	Sweeney <i>et al.</i> , 1985
<i>Amblyospora albifasciati</i>	<i>Aedes albifasciatus</i>	<i>Mesocyclops annulatus</i>	Present study
<i>Amblyospora californica</i>	<i>Culex tarsalis</i> ^b	<i>Mesocyclops leukarti</i>	Becnel <i>et al.</i> , 1992
		<i>Macrocyclus albidus</i>	
<i>Amblyospora dolosi</i>	<i>Culex dolosus</i>	<i>Metacyclus mendocinus</i> ^a	Miceli <i>et al.</i> , 1998
<i>Parathelohania anophelis</i>	<i>Anopheles quadrimaculatus</i>	<i>Microcyclus varicans</i>	Avery and Undeen, 1990

^a Life cycle not completed in the laboratory.

^b Experimental host.

AMENDED TAXONOMIC SUMMARY

Amblyospora albifasciati García and Becnel, 1994.

Amblyospora sp. García, 1989, *Rev. Soc. Entomol. Argent.* **47** (1–4), 100.

Amblyospora albifasciati García and Becnel, 1994, *J. Invertebr. Pathol.* **64**, 243–252.

Type definitive host. *Aedes* (Ochlerotatus) *albifasciatus* (Macquart, 1837). (Diptera, Culicidae).

Type intermediate host. *Mesocyclops annulatus* (Wierzejski, 1892) (Copepoda, Cyclopidae).

Transmission. Transovarially to filial generations of *A. albifasciatus* via binucleate spore. *Per os* to *M. annulatus* via meiospores liberated into environment by death of infected mosquito larvae of filial generation. *Per os* to *A. albifasciatus* via uninucleate spores from *M. annulatus*.

Site of infection. Adipose tissue and ovary of *A. albifasciatus*. Ovary of *M. annulatus*.

Interface. Sporophorous vesicle produced by the sporont during the sporulation sequence involving meiosis in the mosquito host. A nonpersistent sporontogenic interfacial envelope is produced by the sporont in the copepod host. All other stages in direct contact with the host cell cytoplasm.

Development. Uninucleate spores from *M. annulatus* ingested by mosquito larvae initiates schizogonic series that ends with gametes. Gametes unite in pairs (gametogony) and nuclei associate as diplokarya to form meronts. Sporulation in the female ends with binucleate spores that transovarially infect filial generations. Sporulation in filial generation involves meiosis and ends with the production of meiospores. Meiospores ingested by copepod initiate a schizogonic sequence leading to sporulation and production of the

uninucleate spores responsible for horizontal transmission to a new generation of *A. albifasciatus*.

Spores. *Spore from copepod:* $10.4 \pm 0.63 \times 4.8 \pm 0.12 \mu\text{m}$ ($n = 15$, fresh). *Pyriform.* Polar filament isofilar with 9–11 turns, polaroplast extensive and vesiculate. Endospore thicker than the exospore. *Binucleate spore:* oval, $9.27 \pm 0.87 \times 3.10 \pm 0.3 \mu\text{m}$ ($n = 15$, fresh). *Meiospore:* Elongate ovoid, much attenuated anteriorly and slightly attenuated posteriorly. Uninucleate with a thick spore wall and large posterior vacuole. Polaroplast lamellate and more tightly arranged in the apical region. Polar filament anisofilar with 4.5 (range, 4–5) broad proximal and 6.5 (range, 6–8) narrow distal coils all arranged in a single row, $7.2 \pm 0.3 \times 4.4 \pm 0.4 \mu\text{m}$ (fresh, $n = 32$).

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